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Original Article

A UPLC-MS/MS METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF SOFOSBUVIR FROM HUMAN PLASMA

DARSHAN BHATT[a],* B. RAJKAMAL[b]

[a]Mewar University, Gangrar, Chittorgarh 312901, Rajasthan, India, [b]KVK College of Pharmacy, Surmajiguda, Hyderabad 501512, Telangana, India
Email: darshanbhatt1984@gmail.com

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ABSTRACT

Objective: The present work aimed to develop a simple, rapid, specific and precise ultra-performance liquid chromatography-tandem mass spectrophotometric (LC–MS/MS) validated method for quantification of sofosbuvir and internal standard (ISTD) Sofosbuvir-d3 in human plasma.

Methods: Samples prepared by employing liquid-liquid extraction (LLE) using 2.5 ml of ethyl acetate. Chromatographic separation was achieved on Gemini 5µ C18, 50 x 4.6 mm column using a mixture of 0.1% (v/v) formic acid in water to methanol at a ratio of 30:70 v/v as the mobile phase. The flow rate was 0.50 ml/min. The LC eluent was split, and approximately 0.1 ml/min was introduced into Tandem mass spectrometer using turbo Ion Spray interface at 325 °C. Quantitation was performed by transitions of 428.35/279.26 *(m/z)* for sofosbuvir and *431.38/282.37 (m/z)* for sofosbuvir-d3.

Results: The concentrations of ten working standards showed linearity between 4.063 to 8000.010ng/ml ($r^2 \geq 0.9985$). Chromatographic separation was achieved within 2 min. The average extraction recoveries of three quality control concentrations were 75.36% for sofosbuvir and were within the acceptance limits. The coefficient of variation was ≤15% for intra-and inter-batch assays. The %CV of ruggedness ranges 0.35% and 3.09%. The % stability of short term and long term stock solution stability studies was found to be 97.25% and 98.81% respectively.

Conclusion: The results obtained for specificity, linearity, accuracy, precision, ruggedness and stability studies were within the acceptance limits. Thus the validated economical method was applied for pharmacokinetic studies of sofosbuvir.

Keywords: Sofosbuvir, LC-MS/MS, Human plasma, Stability studies

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INTRODUCTION

Sofosbuvir, a phosphoramidate prodrug, is chemically described as (*S*)-Isopropyl 2-((*S*) ((2*R*, 3*R*, 4*R*, 5*R*)-5-(2, 4-dioxo-3,4-dihydro-pyrimidin-1(2*H*)-yl)-4-fluoro-3-hydroxy-4 methyl tetrahydrofuran-2-yl) methoxy)-(phenoxy) phosphorylamino) propanoate [1-2]. Literature survey reveals two HPLC methods for determination of sofosbuvir from its bulk and pharmaceutical dosage forms [3-4]. Three UPLC-MS/MS method were reported for quantification of sofosbuvir from its metabolites and along with other drugs from human plasma [5-7]. Described here is a simple, sensitive, and selective UPLC-MS/MS method for sofosbuvir in the human plasma concentration range of 4.063 to 8000.010ng/ml. As there is no literature on stability and validation details of sofosbuvir estimation from human plasma, this study performed assay validations, according to the FDA guidelines [8]. While this method with validation details were economical and applied for pharmacokinetic studies of sofosbuvir.

MATERIALS AND METHODS [5]

Apparatus and software

The UPLC (Waters, Model Acquity) was coupled with Mass spectrometer (Waters Quattro Premier XE) having Turbo Ion Spray (Waters Quattro Premier XE). The chromatographic integration was performed by MassL ynx V4.1 software.

Chemicals and reagents

Sofosbuvir and Sofosbuvir-d3 (IS) were procured from Mylan Laboratories Ltd, Hyderabad, Formic acid, Methanol and ethyl acetate was procured from Merck Specialities Pvt. Ltd, Mumbai, India. Water used was collected from water purification systems (Milli Q, MilliPore, USA) installed in the laboratory. Pooled drug-free expired frozen human plasma (K2-EDTA as anticoagulant) was obtained froma Blood Bank, Hyderabad, was used during validation and study sample analysis. The plasma was stored into-70±5 °C.

Standards and working solutions

Calibration standard solutions

Stock solutions of sofosbuvir and Sofosbuvir-d3 internal standard (IS) were prepared in methanol. Further dilutions were carried out in 50% methanol. Calibration standards often concentration levels were prepared freshly by spiking drug-free plasma with a sofosbuvir stock solution to give the concentrations of 4.063, 8.125, 62.5, 125.0, 250, 500, 1000, 2000, 4000 and 8000ng/ml.

Quality control standards

Lowest quality control standards, Median quality control standards and highest quality control standards were prepared by spiking drug-free plasma with sofosbuvir to give a solution containing 11.488, 522.180 and 7252.503 ng/ml respectively. They were stored at-20 °C till the time analysed.

Chromatographic conditions

Chromatographic separation was performed on Gemini 5µ C18, 50 x 4.6 mm, analytical column and the mobile phase was a mixture of 0.1% (v/v) formic acid in water to methanol at a ratio of 30:70 v/v. Injection volume was 10µL. The flow rate was 0.50 ml/min. Total analysis time of single injection was 2.0 min. Column oven temperature and autosampler temperature was set to 30 °C and 10 °C, respectively.

Mass spectrometric conditions

The LC eluent was split, and approximately 0.100 ml/min was introduced via electrospray ionisation using a Turbo Ion Spray interface set at 325 °C to generate positive ions [M+H]+. The Mass spectrometric parameters were optimised as shown in table no 1.

Table 1: Mass spectrometric conditions

Capillary voltage	3500V	
Nozzle voltage	1500V	
Delta EMV(+)	500 Positive	
Gas flow	5 L/min	
Gas temperature	350 °C	
Nebulizer pressure	25 psi	
Sheath gas temperature	300 °C	
Sheath gas flow	11L/min	
Acquisition		
Parameters	**Sofosbuvir**	**ISTD**
Transition	428.35/279.26 (m/z)	431.38/282.37 (m/z)
Polarity	Positive	Positive
MS1 resolution	Unit	Unit
MS2 resolution	Unit	Unit
Dwell time (millisec)	200	200
Fragmentor (V)	100	100
Collision energy (V)	8	10

Sample preparation method

To 250 µl of plasma, 50 µl of ISTD (1µg/ml) and 50 µl of 0.1% formic acid was added and vortexed. The drug was extracted with 2.5 ml of ethyl acetate, followed by centrifugation at 2000 rpm/min on a cooling centrifuge for 15 min at 4 °C. The supernatant of 2 ml was withdrawn and evaporated at 50 °C 15 psi of nitrogen until dryness at LV evaporator. The residue was reconstituted with 500 µl of mobile phase, and respective samples were injected into the column.

Validation [9-13]

Specificity

A solution containing 4.063ng/ml was injected onto the column under optimised chromatographic conditions to show the separation of sofosbuvir from impurities and plasma. The specificity of the method was checked for the interference from plasma.

Linearity

Spiked concentrations were plotted against peak area ratios of sofosbuvir to the internal standard and the best fit line was calculated. Wide range calibration was determined by solutions containing4.063 to 8000.010ng/ml.

Recovery studies

The % mean recoveries were determined by measuring the responses of the extracted plasma Quality control samples at HQC, MQC and LQC against un-extracted Quality control samples at HQC, MQC and LQC.

Precision and accuracy

The between-run (Inter-day) accuracy and precision evaluation were assessed by the repeated analysis of human K_3 EDTA plasma samples containing different concentrations of sofosbuvir on separate occasions. A single run consisted of a calibration curve plus six replicates of the lower limit of quantitation, low, medium and high-quality control samples.

Within-run (Intraday) accuracy and precision evaluations were performed by analysing replicate concentrations of sofosbuvir in human K_3 EDTA plasma. The run consisted of a calibration curve plus a total of 24 spiked samples, six replicates of each of the LLOQ, lower, medium and higher quality control samples.

Matrix effect

The matrix effect for the intended method was assessed by using chromatographically screened human plasma. Concentrations equivalent to LLOQ of Sofosbuvir were prepared with seven different plasma batches/lots. Samples were analysed along with one set of freshly spiked CC Standards prepared in the screened biological matrix.

Ruggedness

The ruggedness of the method was assessed by analysing a precision and accuracy batch using a different column, by the different analyst in another instrument.

Stability studies

Short-term stock solution stability of sofosbuvir

Solutions of sofosbuvir were prepared in methanol (Stability Samples) and were kept at room temperature for 6 h 30 min. A freshly prepared solution of sofosbuvir (Comparison Samples) and stability samples were diluted at approximately the same analyte concentration and analysed in a single run; analyte responses were used to determine % stability over time.

Short-term stock solution stability of internal standard

Solutions of internal standard (Sofosbuvir-d3) were prepared in methanol (Stability Samples) and were kept at room temperature for 6 h 30 min. A freshly prepared solution of internal standard (Comparison Samples) and stability samples were diluted at approximately the same analyte concentration and analysed in a single run; Analyte responses were used to determine % stability over time.

Long-term stock solution stability of sofosbuvir

Solutions of Sofosbuvir were prepared in methanol (Stability Samples) and were kept at refrigerator (2-8 °C) for 10 D 02 H. A freshly prepared solution of sofosbuvir (Comparison Samples) and stability samples were diluted at approximately the same analyte concentration and analysed in a single run.

Long-term stock solution stability of internal standard

Solutions of Internal standard were prepared in methanol (Stability Samples) and were kept at refrigerator (2-8 °C) for 10 D 02 H. A freshly prepared solution of internal standard (Comparison Samples) and stability samples were diluted at approximately the same analyte concentration and analysed in a single run.

Freeze-thaw stability

Samples were prepared at low and high-quality control levels, aliquoted and frozen at-70 °C. Some of the aliquots of quality control samples were subjected to five freeze-thaw cycles (stability samples). A calibration curve and quality control samples were freshly prepared (Comparison Samples) and processed with 6 replicates of stability samples and analysed in a single run.

RESULTS AND DISCUSSION

The chromatography observed during the course of validation was acceptable and representative chromatograms of standard blank, HQC, MQC, LQC and LLOQ are shown in fig. 1-3.

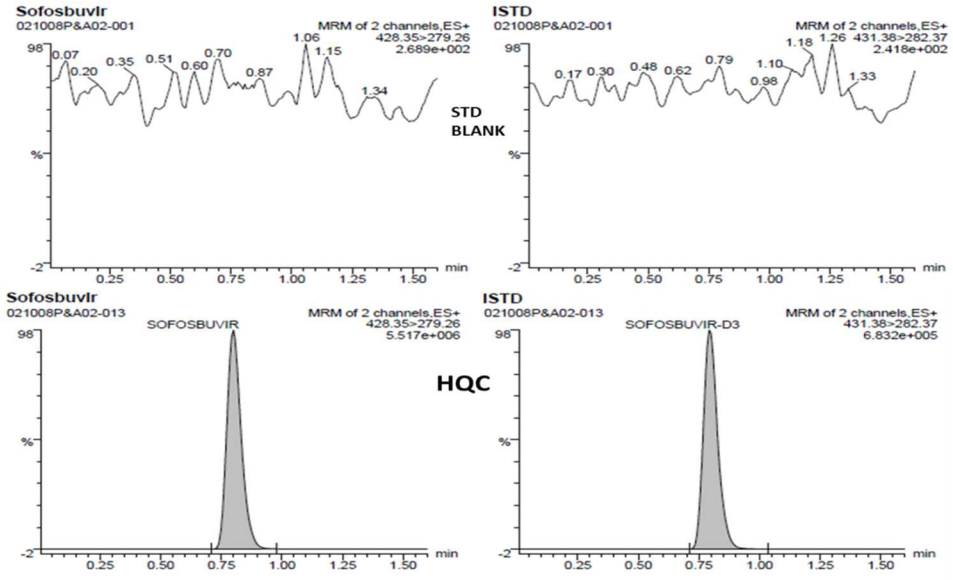

Fig. 1: Chromatograms of standard blank and HQC matrix

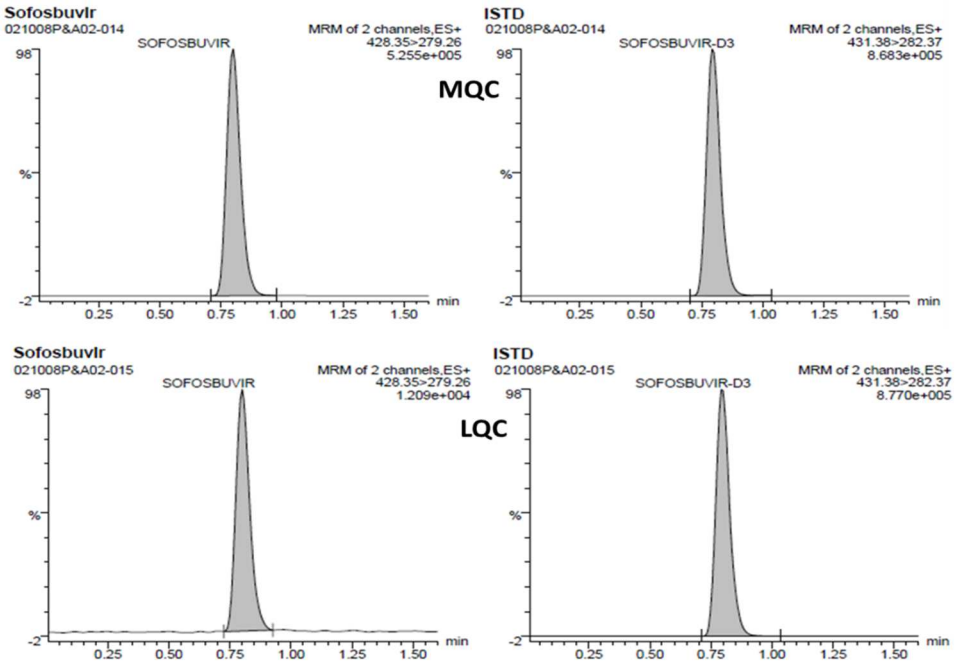

Fig. 2: Chromatograms of MQC and LQC

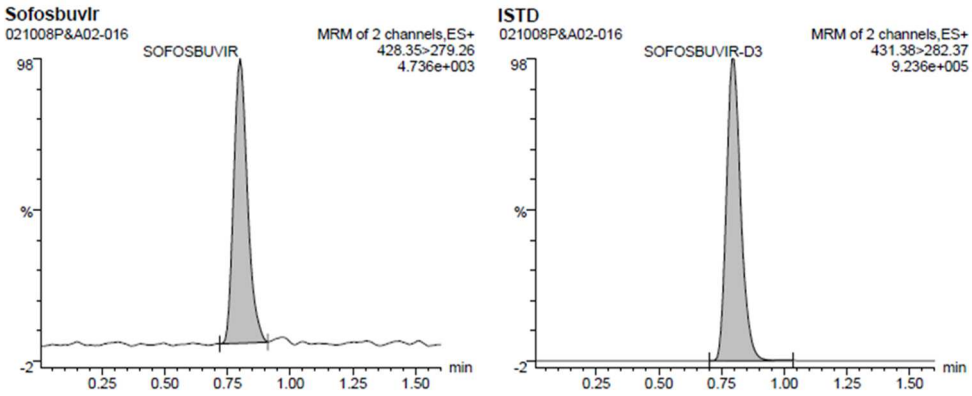

Fig. 3: Chromatograms of LLOQ

The method developed was validated for specificity, accuracy and precision, linearity, ruggedness and stability as per FDA guidance [9-11]. The results of validating parameters are given below.

Specificity

Nine different lots of plasma were analysed to ensure that no endogenous interferences were present at the retention time of sofosbuvir and Sofosbuvir-d3. Nine LLOQ (4.063 ng/ml**)** level samples along with plasma blank from the respective plasma lots were prepared and analysed. (table 2). In all plasma blanks, the response at the retention time of sofosbuvir was less than 20% of LLOQ response and at the retention time of IS, the response was less than 5% of mean IS response in LLOQ. The typical chromatogram of plasma blank and the chromatogram of LLOQ was shown in (fig. 1).

Table 2: Results of specificity for sofosbuvir and sofosbuvir-d3 (ISTD)

S. No.	Drug response				ISTD response			
	STD BL	LLOQ		% Interference	STD BL	LLOQ		% Interference
		Area	RT			Area	RT	
01	0	298	0.800	NIL	0	61776	0.800	NIL
02	0	290	0.800	NIL	0	66613	0.800	NIL
03	0	334	0.800	NIL	0	70621	0.800	NIL
04	0	267	0.807	NIL	0	64807	0.800	NIL
05	0	271	0.800	NIL	0	67694	0.800	NIL
06	0	303	0.800	NIL	0	65249	0.800	NIL
07	0	281	0.800	NIL	0	68774	0.800	NIL
08	0	255	0.800	NIL	0	62927	0.800	NIL
09	0	147	0.800	NIL	0	37012	0.800	NIL
10	0	283	0.800	NIL	0	66641	0.800	NIL

Linearity

The calibration curve (peak area ratio Vs Concentration) was linear over working range of 4.063 to 8000.010ng/ml with ten point calibration used for quantification by linear regression, shown in (fig. 2). The regression equation for the analysis was Y=0.0011227x-0.000164437 with coefficient of correction (r^2) = 0.9985.

Recovery

The % mean recovery for sofosbuvir in LQC, MQC and HQC was 75.47%, 74.37% and 76.26% respectively (table 3).

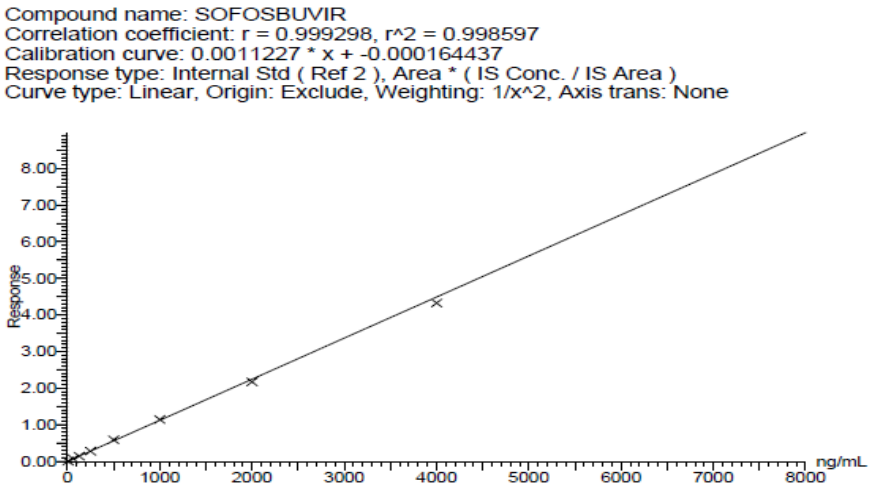

Fig. 3: Spiked concentrations (4.063 to 8000.010ng/ml) were plotted against calculated concentration Vs concentration with ten point calibration used for quantification by linear regression

Table 3: The % mean recovery of sofosbuvir for LQC, MQC and HQC

S. No.	HQC		MQC		LQC	
	Aqueous area ratio	Extracted area ratio	Aqueous area ratio	Extracted area ratio	Aqueous area ratio	Extracted area ratio
01	13.466	8.226	0.981	0.598	0.021	0.013
02	13.541	8.082	1.010	0.590	0.022	0.013
03	13.318	7.995	0.995	0.571	0.021	0.012
04	13.133	8.248	1.001	0.599	0.021	0.013
05	12.997	7.994	0.985	0.600	0.021	0.013
Mean	13.2910	8.1090	0.9944	0.5916	0.0212	0.0128
SD	0.22652	0.12243	0.01178	0.01218	0.00045	0.00045
% CV	1.70	1.51	1.18	2.06	2.11	3.49
% Mean Recovery	76.26		74.37		75.47	
%Global Recovery			75.36			

Intraday (within run) and Inter-day (between run) precision and accuracy

The within-run coefficients of variation ranged between 1.06% and 5.06% for sofosbuvir. The within-run percentages of nominal concentrations ranged between 97.21% and 105.93% for sofosbuvir. Results are presented in table 4.

The between-run coefficients of variation ranged between 2.04% and 5.48% for sofosbuvir. The between-run percentages of nominal concentrations ranged between 98.34% and 100.58% for sofosbuvir. Results are presented in table 4.

Matrix effect

The % accuracy of LLOQ samples prepared with the different biological matrix lots were found within the range of 89.49 to 97.49% which were found within the range of 80.00-120.00% for the seven different plasma lots. % CV for LLOQ samples was observed as 2.87% which are within 20.00% of the acceptance criteria. Results are presented in table 5.

Table 4: Intraday and interday precision and accuracy

QC ID	HQC	MQC	LQC	LLOQ QC
Concentration (ng/ml)	7252.503	522.180	11.488	4.136
Within Batch Precision and Accuracy				
PandA I	Calculated Concentration (ng/ml)			
	6910.342	511.080	11.630	4.290
	7009.484	518.984	10.484	3.998
	7189.506	514.176	11.501	4.116
	7156.740	511.840	11.892	4.132
	6984.985	504.031	11.887	4.477
Mean	7050.211	512.0222	11.4788	4.2026
SD	118.5622	5.42876	0.58102	0.18526
% CV	1.68	1.06	5.06	4.41
% Mean Accuracy	97.21	98.05	99.92	101.61
PandA II	7234.610	533.688	12.086	4.263
	7192.185	531.929	12.605	4.266
	7272.508	523.890	12.009	4.246
	7351.433	522.452	11.705	4.070
	7380.960	535.319	12.440	4.172
Mean	7286.339	529.4556	12.1690	4.2034
SD	78.93435	5.88296	0.35753	0.08377
% CV	1.08	1.11	2.94	1.99
%	100.47	101.39	105.93	101.63
Mean Accuracy				
PandA III	7161.887	520.892	11.414	4.123
	7036.505	514.024	11.006	4.395
	6960.208	497.103	10.554	4.354
	7181.121	521.290	11.095	4.168
	6960.064	522.273	11.006	4.342
Mean	7059.957	515.1164	11.0150	4.2764
SD	106.7109	10.58730	0.30752	0.12214
% CV	1.51	2.06	2.79	2.86
% Mean Accuracy	97.35	98.65	95.88	103.39
Between Batch Precision and Accuracy				
Mean	7132.169	518.8647	11.5543	4.2275
SD	147.64818	10.58931	0.63313	0.13174
% CV	2.07	2.04	5.48	3.12
% Mean Accuracy	98.34	99.37	100.58	102.21

Table 5: Results of matrix effect

LLOQ nominal concen (4.063ng/ml)		
S. No.	**Calculated LLOQ concn (ng/ml)**	**% accuracy**
1	3.937	96.9
2	3.808	93.73
3	3.823	94.09
4	3.961	97.49
5	3.636	89.49
6	3.867	95.17
7	3.766	92.69
	% Mean accuracy	94.223
	SD	2.7003
	% CV	2.87

Ruggedness

The coefficients of variation ranged between 0.35% and 3.09% for sofosbuvir. The percentages of nominal concentrations ranged between 93.2% and 99.29% for sofosbuvir. Results are presented in table 6.

Stability studies

Short-term stock solution stability of sofosbuvir and internal standard

Sofosbuvir and internal standard were found to be stable in methanol for 6 h 30 min at room temperature with a % stability of 97.25% and 97.0% respectively. Results are presented in table 7.

Long-term stock solution stability of sofosbuvir and internal standard

Sofosbuvir and internal standard were found to be stable in methanol 10 D 02 H at refrigerator (2-8 °C) with a % stability of 98.81% and 107.96% respectively. Results are presented in table 8.

Freeze-thaw stability

Sofosbuvir is found to be stable in human K_3 EDTA plasma after five freeze-thaw cycles at-70 °C with coefficients of variation of 3.27% (LQC) and 3.86% (HQC) for sofosbuvir, and the percentages of nominal concentrations for sofosbuvir were found to be 103.17% (LQC) and 101.23% (HQC). Results are presented in table 9.

Table 6: **Results of ruggedness with different column**

QC ID	HQC	MQC	LQC	LLOQ QC
Conc.(ng/ml)	7252.503	522.180	11.488	4.136
PandA ID	Calculated concentration (ng/ml)			
Different	Acquisition batch ID: 031008PandADC01			
Column	6980.672	523.650	11.419	4.044
	7005.431	518.262	11.463	3.725
	7243.518	521.038	11.403	3.853
	7100.206	527.007	11.307	3.861
	7312.115	516.714	11.443	3.790
Mean	7128.3884	521.3342	11.4070	3.8546
SD	145.55342	4.13570	0.06040	0.11925
% CV	2.04	0.79	0.53	3.09
% Mean Accuracy	98.29	99.84	99.29	93.20

Table 7: Short-term stock solution stability of drug and ISTD

S. NO.	Drug		ISTD	
	Nominal Conc (ng/ml)		Nominal Conc (µg/ml)	
	396675.19	400000.4	4.034	4.075
	Area ratio		Area ratio	
	comparison samples	Stability samples	Comparison samples	Stability samples
01	9.134	9.076	0.116	0.115
02	9.181	8.829	0.117	0.114
03	9.147	9.090	0.115	0.117
04	9.082	8.973	0.117	0.113
05	9.231	8.946	0.114	0.111
06	9.197	8.996	0.117	0.112
Mean	9.1620	8.9850	0.1160	0.1137
SD	0.05245	0.09532	0.00126	0.00216
% CV	0.57	1.06	1.09	1.90
% Mean Stability	97.25		97.00	

Table 8: Long-term stock solution stability of drug and internal standard

S. No.	DRUG		ISTD	
	Nominal Conc (ng/ml)		Nominal Conc (µg/ml)	
	400000.480	398186.240	4.214	4.075
	Area ratio		Area ratio	
	Comparison samples	Stability samples	Comparison samples	Stability samples
01	9.219	9.049	0.108	0.111
02	9.116	9.111	0.107	0.110
03	9.228	9.026	0.108	0.115
04	8.918	9.141	0.112	0.119
05	9.208	9.073	0.111	0.119
06	9.138	9.022	0.113	0.114
Mean	9.1378	9.0703	0.1098	0.1147
SD	0.11700	0.04777	0.00248	0.00383
% CV	1.28	0.53	2.26	3.34
% Mean Stability	98.81		107.96	

Table 9: Freeze-thaw stability at-70 °C

S. No.	HQC		LQC	
	Nominal Conc (ng/ml)		Nominal Conc (ng/ml)	
	7252.503		11.488	
	Calculated Conc (ng/ml)	% accuracy	Calculated Conc (ng/ml)	% accuracy
1	7255.363	100.04	11.571	100.72
2	6985.35	96.32	11.547	100.51
3	7017.724	96.76	12.168	105.92

CONCLUSION

Chromatographic separation was performed on Gemini 5µ C18, 50 x 4.6 mm, analytical column and the mobile phase was a mixture of 0.1% (v/v) formic acid in water to methanol at a ratio of 30:70 v/v. The drug was extracted from the sample with 2.5 ml of ethyl acetate. The specificity of the method was checked for the interference from plasma. Wide range calibration was determined by solutions containing 4.063 to 8000.010ng/ml. The % mean recovery for sofosbuvir in LQC, MQC and HQC was 75.47%, 74.37% and 76.26% respectively. The within-run coefficients of variation ranged between 1.06% and 5.06% for sofosbuvir. The between-run coefficients of variation ranged between 2.04% and 5.48% for sofosbuvir the % accuracy of LLOQ samples prepared with the different biological matrix lots were found within the range of 89.49 to 97.49%. Stability test were performed to assess the long term and short term stability of sofosbuvir sample solutions,

internal standard solutions. The developed method was validated for the quantitative determination of sofosbuvir from plasma was simple, rapid, specific, sensitive, accurate and precise. Hence, the method is quite suitable to detect the drug from plasma samples of human volunteers.

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CONFLICT OF INTERESTS

Declared none

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